

CD9, a Major Platelet Cell Surface Glycoprotein, Is a ROCA Antigen and Is Expressed in the Nervous System

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We previously generated a monoclonal antibody (mAb), ROCA1, which binds preferentially to rostral versus caudal sympathetic ganglia and intercostal nerves. Two other mAbs, ROCA2 and B2C11, bind to the same structures but not in rostrocaudal gradients. All three mAbs recognize a 26 kDa cell surface protein. Amino acid sequence data obtained from the affinity purified 26 kDa protein showed some homology with human CD9, a tetraspan protein implicated in intercellular signaling in hematopoietic cells. Using the PCR, we obtained cDNA clones representing the entire rat CD9 coding sequence from sciatic nerve and sympathetic ganglia. ROCA1, ROCA2, and B2C11 each immunoprecipitate a 26 kDa protein from CHO cells stably transfected with one of the clones, demonstrating that the ROCA cell surface antigen is indeed rat CD9. We find that CD9 mRNA is widely expressed, with particularly high levels present in a number of neural tissues. *In situ* hybridization demonstrates that peripheral neurons and Schwann cells, as well as adrenal chromaffin cells express CD9 mRNA. Consistent with immunoblot analyses showing that, unlike the ROCA1 epitope, the 26 kDa protein is not expressed in a rostrocaudal gradient, we find similar levels of rat CD9 mRNA in rostral and caudal intercostal nerves. In developing postnatal rat sciatic nerve, CD9 mRNA levels are coordinately regulated with the expression of myelin genes. These results provide another example of a cell surface protein expressed by both hematopoietic and neural cells, and suggest a role for CD9 in intercellular signaling in the nervous system.

[Key words: CD9, tetraspan family, ROCA antigen, neurospecification, Schwann cell, platelets, adrenal chromaffin cells, myelin, cell surface]

Cell surface proteins play fundamental roles in cell–cell interactions in the nervous and hematopoietic systems. In the hematopoietic system, some of these roles include platelet activation and aggregation, lymphocyte trafficking, and tumor cell motility. In the nervous system, cell surface proteins play key roles in neuron–neuron and neuron–glia interactions, and direct

the migration of neurons and glia, as well as axon outgrowth, guidance, and fasciculation. Many of these proteins belong to four major cell adhesion receptor families: cadherins, Ig superfamily, integrins, and selectins (Hynes and Lander, 1992). Most of these proteins possess single transmembrane domains or are linked to the membrane via a phosphoinositol anchor, and they contain one or more Ig domains, an EGF or fibronectin repeat, and a cysteine-rich repeat or a kinase domain. Hematopoietic and neural cells express members of each family, with the exception of the selectins, which have thus far been identified only in hematopoietic cells.

A new superfamily of cell surface proteins known as the tetraspan (Gil et al., 1992) or transmembrane 4 superfamily (TM4SF; Wright et al., 1993) has recently been identified. Members of this family include (1) the leucocyte proteins CD9 (Boucheix et al., 1991; Lanza et al., 1991; Martin-Alonso et al., 1992; Mitamura et al., 1992; Rubinstein et al., 1993a,b), MRP-1 (identical to human CD9; Miyake et al., 1991), CD37 (Classon et al., 1989), CD53 (Amiot, 1990; Angelisova et al., 1990), OX-44 antigen (rat homolog of human CD53; Bellacosa et al., 1991), CD63/ME 491 (Hotta et al., 1988; Metzelaar et al., 1991), AD1 (rat homolog of human CD63/ME 491; Nishikata et al., 1992), R2/C33/IA4 (Gaugitsch et al., 1991; Imai et al., 1992; Gil et al., 1992, respectively), TAPA-1 (Oren et al., 1990; Andria et al., 1991), and A15 (Emi et al., 1993); (2) the tumor-associated antigens CO-029 (Szala et al., 1990) and L6 (Marken et al., 1992); (3) the lung epithelial protein TI-1 (Kallin et al., 1991); and (4) the schistosome surface proteins Sm23 (Wright et al., 1990) and Sj23 (Davern et al., 1991). The current model for the structure of superfamily members consists of four transmembrane domains divided by two extracellular loops, with cytoplasmic N- and C-termini (Horejsi and Vlcek, 1991; Levy et al., 1991; Reynolds et al., 1992; Tomlinson et al., 1993). While many of these proteins are known to be expressed in hematopoietic and a variety of other cells, little information is available on their distribution in the nervous system (Kemshead et al., 1982; Komada et al., 1983; Ross et al., 1986; Von dem Borne et al., 1989; Oren et al., 1990). Although the *in vivo* function of these proteins has yet to be elucidated, antibody perturbation experiments implicate certain tetraspan family members in neural cell adhesion, motility, and growth regulation (Anton et al., 1994; Hadjiargyrou and Patterson, 1994).

We previously demonstrated that the ROCA1 mAb recognizes two distinct proteins (60 kDa and 26 kDa) in membrane/cytoskeletal fractions of peripheral nerves and ganglia. The 60 kDa protein is expressed at higher levels in rostral than in caudal intercostal nerves, and has been identified as peripherin (Kaprielian and Patterson, 1993). In contrast, it is the ROCA1

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epitope on the 26 kDa protein, and not the protein itself, that is preferentially visualized immunohistochemically in rostral nerves and ganglia (Suzue et al., 1990; Kaprielian and Patterson, 1993). We now have three mAbs against the 26 kDa protein, ROCA1, ROCA2 (Kaprielian and Patterson, 1993; Tole and Patterson, 1993), and B2C11 (Akeson and Warren, 1984). Amino acid sequence data obtained from the affinity purified 26 kDa protein raised the possibility that it could be the rat homolog of human CD9 (Kaprielian and Patterson, 1993). Here we describe the isolation of cDNA clones encoding rat CD9, confirm that it is the ROCA surface antigen, and define the regions of CD9 that contain the epitope for each mAb. In addition, we report the tissue, cell type, positional, and developmental expression of rat CD9 mRNA.

Materials and Methods

Antibodies

ROCA1 and ROCA2 are both IgG2b mouse mAbs that have been previously characterized (Suzue et al., 1990; Kaprielian and Patterson, 1993; Tole and Patterson, 1993). B2C11 is an IgG2a mouse mAb that has also been previously characterized (Akeson and Warren, 1984).

Protein preparations and immunoblotting

NP40 extracts of adult rat peripheral nerve membrane/cytoskeletal fractions were prepared as previously described (Kaprielian and Patterson, 1993). Platelet extracts were derived from membrane/cytoskeletal fractions of platelet rich plasma, prepared according to Boucheix et al., 1983. In brief, blood from freshly killed adult rats was first collected into 1/9 volume of 0.13 M sodium citrate, and then centrifuged at 1000 rpm ($150 \times g$) for 10 min in a Sorvall SS 34 rotor. The resulting pellet was discarded, while the supernatant, which contains the platelet-rich plasma (PRP), was then centrifuged at $100,000 \times g$ for 1 hr in a Beckman 70 Ti rotor. The pellets were resuspended in protease inhibitor-containing low-salt homogenization buffer (Kaprielian and Patterson, 1993). This material, which will be referred to as the platelet membrane/cytoskeletal fraction, was either used immediately or frozen at -80°C until further use. Detergent extracts were obtained by solubilizing the membrane/cytoskeletal fraction with 1% NP40 as previously described (Kaprielian and Patterson, 1993). Protein concentration was determined by the method of Lowry et al. (1951), and immunoblots were performed as previously described (Kaprielian and Patterson, 1993).

Molecular cloning of rat CD9

Single strand cDNA was prepared from adult rat SCG and ScN RNA using Maloney murine leukemia virus reverse transcriptase (Promega) and oligo-dT primers. Two primers, NT17 and CT19, representing portions of the N- and C-terminus of human/bovine CD9, respectively, were synthesized as follows: NT17, 5'-ATGCCGGTCAAAGGAGG-3'; CT19, 5'-AAGCTTGACTCTAGACCAT-3'. Rat CD9 DNA was amplified by adding these two primers and Taq DNA polymerase (Promega) to SCG or ScN cDNA, and carrying out the following polymerase chain reaction (PCR): 40 cycles of 95°C for 1 min, 52°C for 1.5 min, 72°C for 1.5 min, and a final incubation at 72°C for 10 min. The PCR products amplified from both the SCG and ScN templates each contained a major band of the expected size (678 bp) on 2% agarose gels. These products were isolated and inserted into the SmaI site of the pBluescript II SK+ (Stratagene) vector.

DNA sequence analysis

Two, independent subclones derived from each of the original PCR products named [Full ScN #13 and Full ScN #42 (from ScN cDNA), and Full SCG #3 and Full SCG #8 (from SCG cDNA)] were sequenced by the dideoxy chain termination method (Sanger et al., 1977) using double-stranded DNA as template and T7 DNA polymerase (Sequenase, U.S. Biochemicals). Both strands of each subclone were sequenced not only with the T3, SK, KS, and T7 primers (Stratagene), but also with the four internal primers: RCD9 189, 5'-GCTGGGGCCCTCATGATG-3'; RCD9 255, 5'-CTGGGATTGTTCTTCGGA-3'; RCD9 490, 5'-GAACAAGGATGAGCCAG-3'; RCD9 550, 5'-GACATCTGCCCCAAAAG-3'. The sequences of these subclones proved to be

identical except that at each of four positions one of the clones contained a nucleotide that was different from the one present in the other three. This analysis yielded a consensus nucleotide sequence for rat CD9 (see Fig. 2). Eight additional PCR-isolated cDNAs (four each from ScN and SCG RNA) were sequenced in one direction using the primers listed above. Six of these proved to be identical to the consensus rat CD9 sequence, while two contained single nucleotide differences. We attribute all of the single nucleotide changes to PCR errors. Searches for related sequences were performed using the BLAST program.

Expression of rat CD9 in CHO cells

Subcloning of CD9 cDNA. The Full ScN #13 cDNA was inserted into the pBluescript II SK+ vector (described above). Digestion with BamHI and XhoI yielded a fragment containing all of the CD9 cDNA and approximately 50 bp of vector. This fragment was then subcloned into the BamHI/XhoI linearized eukaryotic expression vector, pcDNA1/neo (Invitrogen). After transformation of bacteria, a plasmid with the intact 5'-3' CD9-cDNA insert was selected. Large amounts of this DNA were purified (Qiagen plasmid kit) and subsequently used for transfection.

Transfection. CHO cells were transfected by lipofectin (BRL) (Felgner et al., 1987). Briefly, cells were plated at $2.5 \times 10^5/100$ mm dish and grown overnight at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Hyclone) and penicillin-streptomycin (50 U/ml and 50 $\mu\text{g}/\text{ml}$, respectively). Lipofectin (25 μg) was mixed with 5 μg rat CD9 cDNA (construct) in 200 μl of serum-free DMEM and added to the cells in 5 ml of serum-free medium. The cells were then incubated for 5 hr after which the lipofectin-DNA medium was replaced by 10 ml of serum-containing DMEM. Cells were finally incubated at 37°C for 48 hr prior to the addition of 400 $\mu\text{g}/\text{ml}$ G418. Twelve days later colonies were isolated using cloning cylinders.

Fusion protein analysis. The MBP-CD9 fusion protein was generated by in-frame fusion to a maltose binding protein (New England Biolabs). Briefly, to obtain a cDNA encoding the large, putative extracellular domain of rat CD9, a PCR was carried out using a primer corresponding to amino acids 111 to 116 (ATGGATCCCACAAGGACGAGGTGAT-TAA), and another corresponding to amino acids 184–190 (ATAAGCTTCACTTGCTGTGGAAGACCTC), along with Full ScN #13 as a template. To facilitate the cloning, a BamHI site was incorporated into the 5' end of the forward primer (underlined), and a HindIII site was incorporated into the 5' end of the backward primer (underlined). The PCR product was extracted with phenol and chloroform, digested with BamHI and HindIII, and ligated with pMal-c2 vector that had been digested with BamHI and HindIII. The expression of this fusion protein was induced by IPTG (isopropyl- β -D-thiogalactopyranoside), and sonicated *E. coli* extracts were subjected to SDS-PAGE (Smith and Johnson, 1988).

Immunoprecipitation. Stably transfected CHO cell lines were grown to confluency in 100 mm tissue culture dishes containing DME-10% FBS. For metabolic labeling, the medium was replaced with 5 ml of DME (without cysteine or methionine or serum) to which 500 μCi (final concentration = 100 $\mu\text{Ci}/\text{ml}$) ^{35}S -methionine-translabel (ICN) had been added, and the cells were then incubated for 3 hr at 37°C . Following the labeling period, cells were washed $3 \times$ with 5 ml of phosphate-buffered saline (PBS; 0.9% NaCl, 100 mM NaPO_4) and lysed with 2 ml of protease-inhibitor containing low salt homogenization buffer (see above) containing 1% CHAPS (Boehringer Mannheim). After multiple rounds of trituration and vortexing, the cell extract was centrifuged at $100,000 \times g$ for 1 hr in a Beckman Type 50 rotor. The resulting supernatant (2 ml) was mixed with 150 μl of Protein G-conjugated sepharose beads (Pharmacia) and rotated end-over-end for 3 hr at room temperature. Then, 500 μl of the preabsorbed extract was mixed with 500 μl of ROCA1, ROCA2, or B2C11 hybridoma supernatant and rotated end-over-end for 12–16 hr at 4°C . After this incubation, 30 μl of Protein G-conjugated sepharose beads were added to the mixture and the incubation was continued for another 3 hr at room temperature. The beads were then centrifuged in a microfuge, the supernatant removed, and the beads washed three times with PBS containing 0.05% Tween-20 by a series of resuspensions and centrifugations. After a final wash in PBS without detergent, the beads were placed in $1 \times$ nonreducing sample buffer for 5 min to elute bound antigens (Kaprielian and Patterson, 1993). Proteins were subjected to one-dimensional SDS-PAGE, and stained and destained as previously described (Kaprielian and Patterson, 1993). Immediately prior to drying, the gels were incubated in

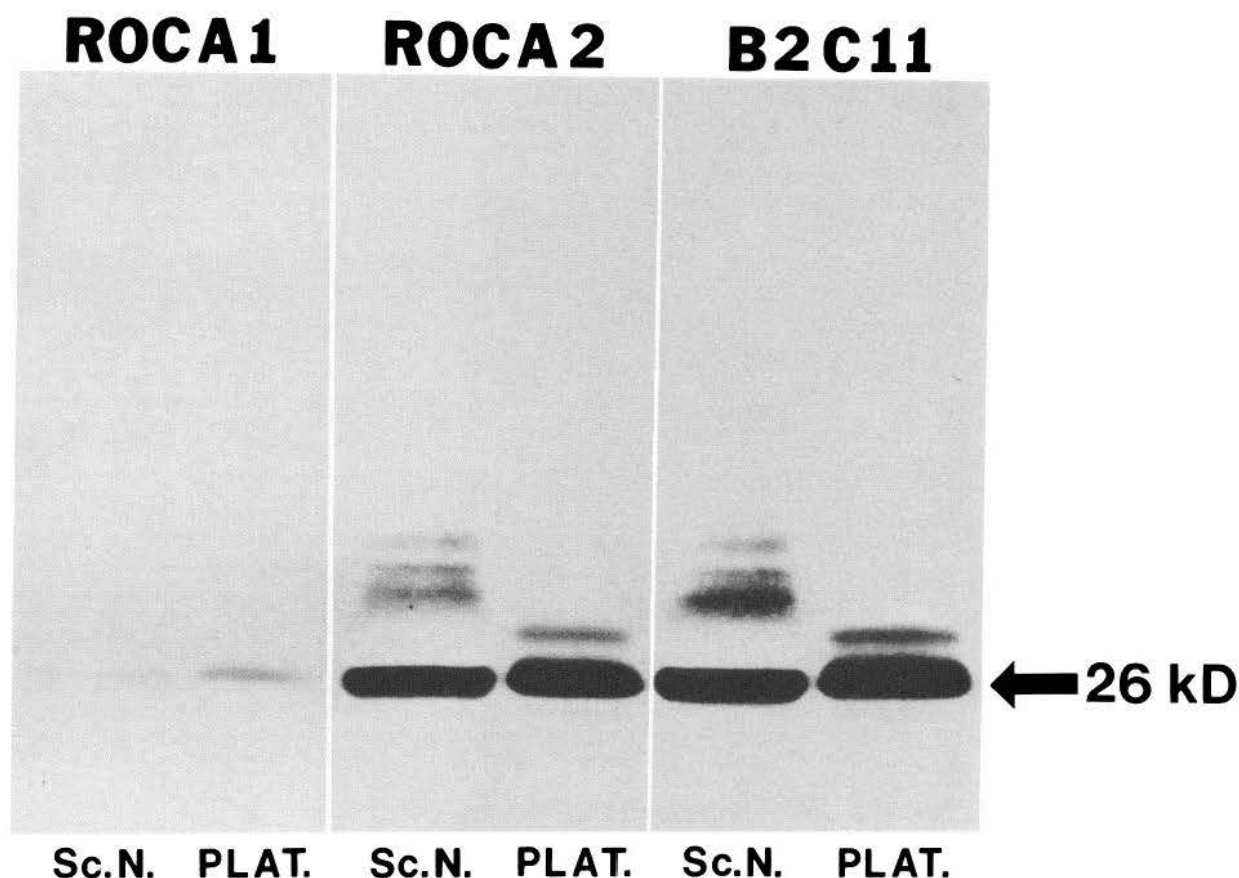


Figure 1. ROCA1, ROCA2, and B2C11 each bind a 26 kDa protein in extracts of adult rat sciatic nerves and platelets. One percent NP-40 extracts of adult rat sciatic nerve and platelet membrane/cytoskeletal fractions were subjected to SDS-PAGE and immunoblot analysis as described in Materials and Methods. Each mAb binds a 26 kDa protein in both sciatic nerve (Sc.N.) and platelet (PLAT.) extracts. Primary antibody binding was visualized with a peroxidase-conjugated goat anti-mouse IgG secondary antibody. Approximately 40 μ g of protein was loaded in each lane.

Amplify (Amersham) for 30 min at room temperature. For autoradiography, dried gels were exposed to Kodak X-OMAT AR film for 12–24 hr at -80°C .

Immunocytochemistry. Transfected CHO cells were harvested, replated on sterile glass coverslips in serum-containing DMEM and grown for another 48 hr. Surface labeling of the cells with ROCA2 and B2C11 hybridoma supernatants was carried out as described in Hadjiargyrou and Patterson, 1994. Cells were photographed using a Nikon fluorescence microscope (Diaphot 300).

RNA Isolation and Northern analysis. Total RNA was prepared from various tissues as described by (Chomczynski and Sacchi 1987). RNA was separated on 1.5% agarose-formaldehyde gels, transferred to Hybond nylon membrane (Amersham), and UV cross-linked (Stratagene). The PCR was used to generate CD9, MBP and P_0 DNA by using the cloned cDNAs as templates, along with universal and reverse PCR primers. DNA for the 3' end of 28S rRNA was amplified using rat genomic DNA as the template and the following two PCR primers: forward, TGAAGCAGAAATTCACCAAGC; backward, GTGAGGGCTGACTTTCAAT (Choi, 1985). Each of the DNA probes were labeled with ^{32}P by the random priming method (Feinberg and Vogelstein, 1983). Blots were hybridized and washed according to Cho et al., 1991. The same blot was reused multiple times by placing the filter in boiling water for 1 min to strip previously bound radioactivity. Blots were exposed to either Kodak X-OMAT AR film or Molecular Dynamics Phosphor screens. Radioactivity was quantitated by scanning the Phos-

phor screens with a Phosphorimager 400S (Molecular Dynamics) and computations performed with IMAGEQUANT software.

In situ hybridization. The Full ScN #13 cDNA was linearized with BamHI or EcoRI and transcribed with T7 or T3 RNA polymerase (MEGAscript Transcription Kit, Ambion) to generate antisense and sense rat CD9 RNA probes, respectively. Nonradioactive labeling was achieved by incorporating digoxigenin-11-UTP (Boehringer Mannheim) into the RNA probes during the transcription reaction. Tissue sections (15 μm thick) on glass slides were hybridized in plastic slide mailers with digoxigenin-labeled probes by a modification (Birren et al., 1993) of the procedure of Harland (1991) with the following exceptions. All tissue sections were placed on untreated Super frost/Plus microscope slides (Fisher Scientific). Prehybridization and hybridization steps were carried out at 50°C (instead of 60°C) for those sections containing sciatic or intercostal nerves. The alkaline phosphatase-conjugated anti-digoxigenin Fab fragments were preabsorbed with extracts of tissue parts dissected from developing or adult rats, depending on the age of the tissue to be examined.

Results

Expression of the ROCA surface antigen in platelets

To determine whether the ROCA mAbs recognize a 26 kDa protein in hematopoietic cells we performed immunoblots on

Figure 2. *Top*, Amino acid sequence alignment of RAT, MOUSE, HUMAN, MONKEY, and BOVINE CD9. The putative transmembrane domains (TMI–IV) are shaded. Identical residues between sequences are indicated by dashes. *Bottom*, Proposed topological model of CD9. Open circles represent residues that are identical across species. Solid circles identify interspecies amino acid differences. The two lightly shaded circles identify the positions of the two amino acids that are missing in rat CD9 (see top). The asterisk denotes the asparagine residue that is a possible site for N-linked glycosylation.

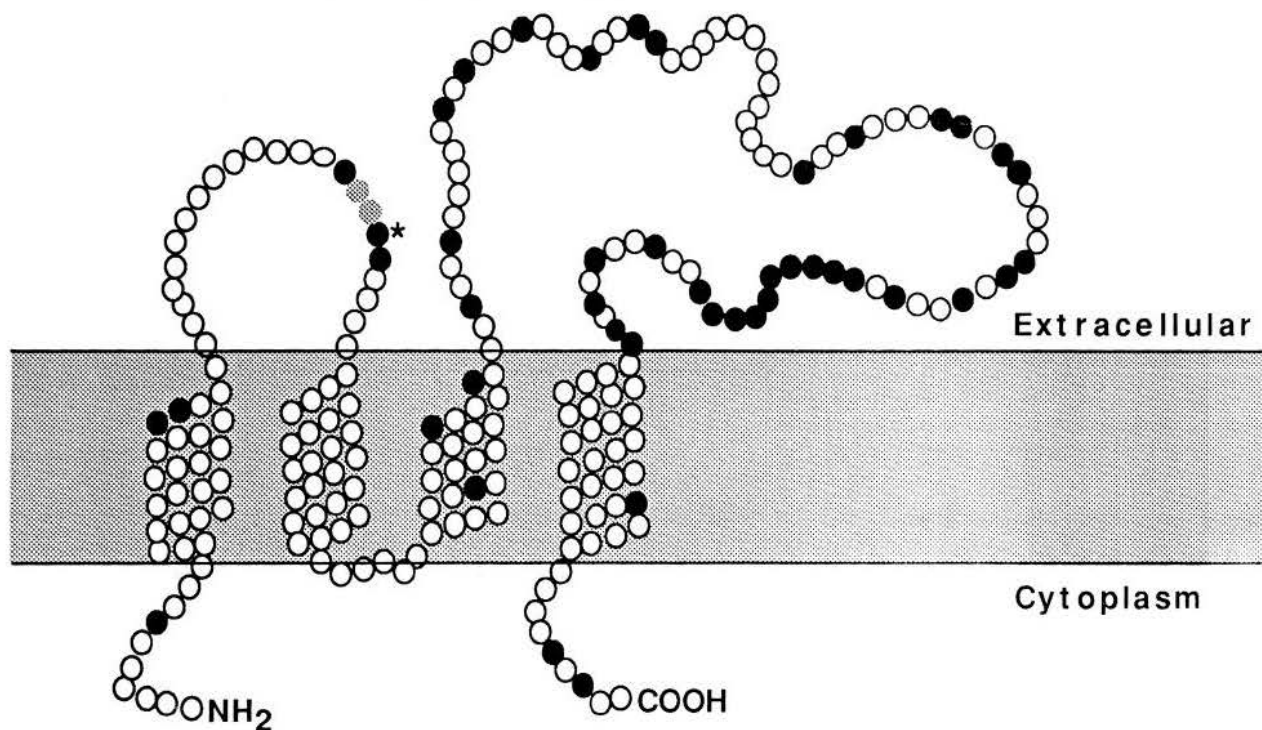
	1	TMI				50
RAT	MPVKGGSKCI	KYLLFGFNFI	FWLAGI AVL A	IGLWLR FDSQ	TKSIFEQET*	
MOUSE	-----S---			A I-----	-----**	
HUMAN	-----T---			A I-----	-----TN	
MONKEY	-----T---			A I-----	-----TN	
BOVINE	-----T---			S V-----	-----**	

	51	TMII			TMIII	100
RAT	*NHSSFYTGV	YILIGAGALM	MLVGFLGCCG	AVQESQCMLG	LFFGFLLVIF	
MOUSE	NNH-----				-G-----	
HUMAN	NNN-----				-G-----	
MONKEY	NNN-----				-G-----	
BOVINE	NND-----				-S-----	

	101					150
RAT	AIEIAAAVWG	YTHKDEVIKE	LQEFYKDTYQ	KLRNKDEPQR	ETLKAIHMAL	
MOUSE	---I---V---	-T--D-----	L-E--K---Q	--RS-----	-----M--	
HUMAN	---I---I---	-S--D-----	V-E--K---N	--KT-----	-----Y--	
MONKEY	---I---I---	-S--D-----	V-E--K---N	--KT-----	-----Y--	
BOVINE	---V---I---	-S--E-----	V-K--E---N	--KN-----	-----I--	

	151					200
RAT	NCCGIAGGVE	QFISDICPKK	QVLESFQVKS	CPDAIDEVFH	SKFHIIGAVG	
MOUSE	D---IA-PL-	--IS-T--K-	QLLESFQVKP	--E--S-V-N	N-----	
HUMAN	N---LA-GV-	--IS-I--K-	DVLETFTVKS	--D--K-V-D	N-----	
MONKEY	D---LA-GV-	--IS-I--K-	DVLETFTIKS	--D--K-V-D	N-----	
BOVINE	D---LT-VP-	--LT-T--P-	NLIDSLKTRP	--E--D-I-R	S-----	

	201	TMIV	228
RAT	IGIAVVMIFG	MIFSMILCCA	IRRSREMV
MOUSE	-----	-I-----	---S-E--
HUMAN	-----	-I-----	---N-E--
MONKEY	-----	-I-----	---N-E--
BOVINE	-----	-V-----	---N-D--



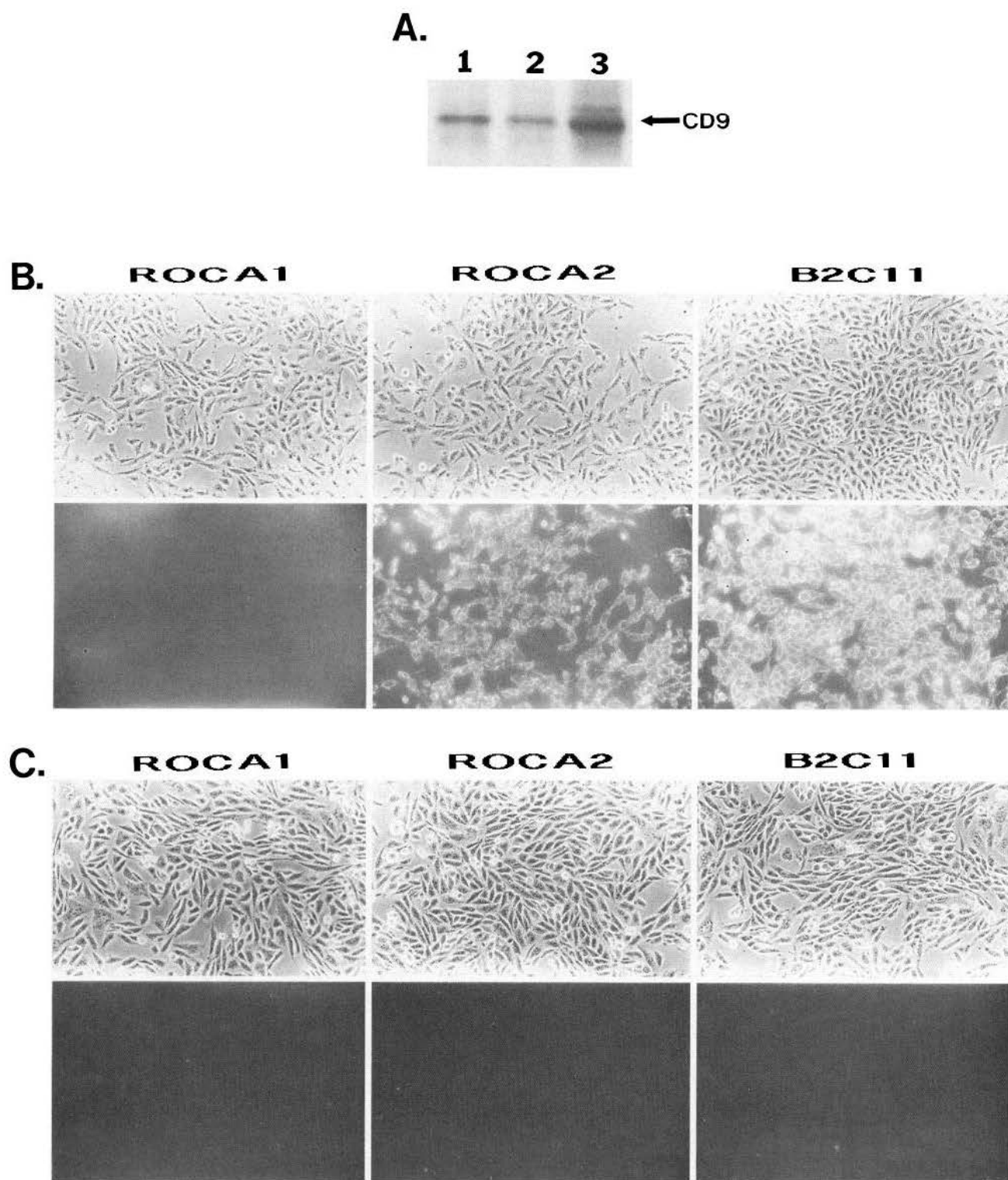


Figure 3. The 26 kDa ROCA cell surface antigen is rat CD9. *A*, ROCA1 (lane 1), ROCA2 (lane 2), and B2C11 (lane 3) each immunoprecipitate a 26 kDa protein from a 1% CHAPS extract of ^{35}S -Met labeled CHO cells that had been stably transfected with the Full ScN. #13 cDNA, encoding rat CD9. The stably transfected CHO cell clone used in this particular experiment was CD9 γ . The arrow indicates the position of CD9 (26 kDa). *B*, ROCA2 and B2C11, but not ROCA1 label the surfaces of CD9 γ cells. Live CD9 γ cells were sequentially incubated with the various mAbs and a goat anti-mouse IgG fluorescein-conjugated secondary antibody. The top portion of each panel is a phase micrograph, while the bottom portion is a fluorescence micrograph of the same field. *C*, ROCA1, ROCA2, and B2C11 do not label the surfaces of nontransfected CHO cells. Live CHO cells were sequentially incubated with the various mAbs and a goat anti-mouse IgG fluorescein-conjugated secondary antibody. The top portion of each panel is a phase micrograph, while the bottom portion is a fluorescence micrograph of the same field.

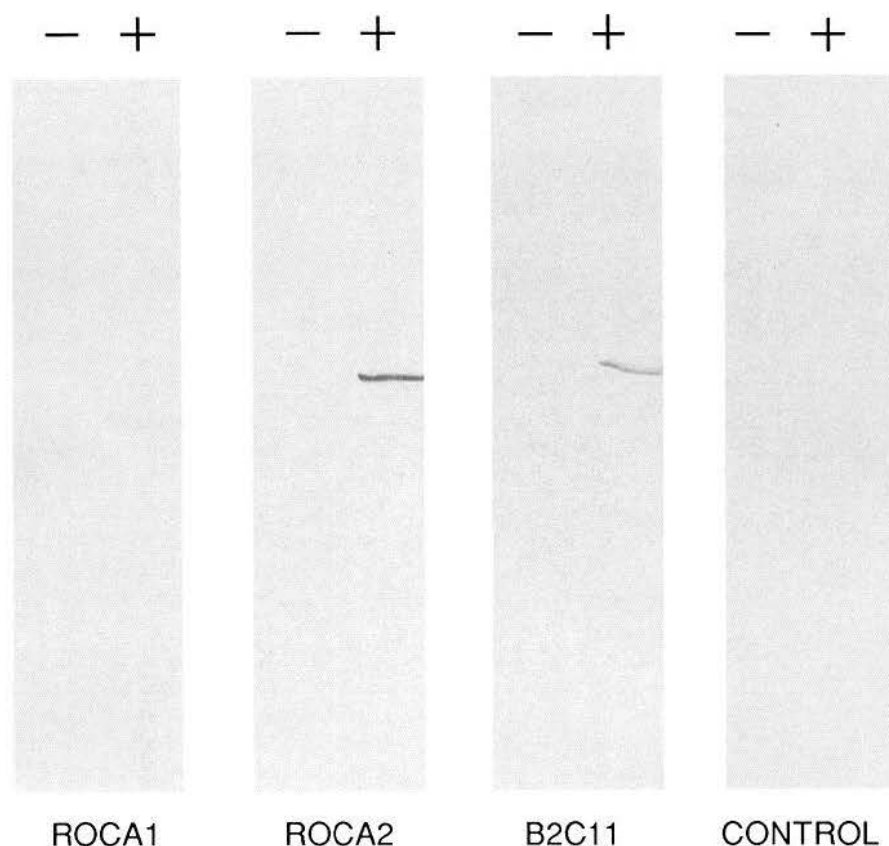


Figure 4. ROCA2 and B2C11 bind the large, putative extracellular domain of rat CD9. Proteins within extracts generated from *E. coli* expressing the MBP-CD9 fusion protein (+) or MBP (–) were subjected to SDS-PAGE and immunoblot analysis. ROCA2 and B2C11, but not ROCA1, bind a 49 kDa protein present only in extracts containing the MBP-CD9 fusion protein. Primary antibody binding was visualized with a peroxidase-conjugated goat anti-mouse IgG secondary antibody. The control reflects the binding of the secondary antibody alone.

detergent-solubilized membrane/cytoskeletal fractions of adult rat platelets using ROCA1 and ROCA2. We also used mAb B2C11 (Akeson and Warren, 1984) after finding that this mAb binds strongly to the affinity-purified 26 kDa ROCA1/2 antigen from adult rat peripheral nerves (data not shown; mAb B2C11 is further characterized in the accompanying article; Hadjiargyrou and Patterson, 1994). Each mAb recognizes a 26 kDa protein in the platelet extract that comigrates with the previously described peripheral nerve protein (Fig. 1). In addition, ROCA2 and B2C11 each bind larger, glycosylated forms of the 26 kDa protein (Kaprielian and Patterson, 1993) in both platelet and nerve extracts. These results are consistent with the possibility that the 26 kDa ROCA antigen may be rat CD9.

Isolation of rat CD9 cDNA clones

To clone the cDNA encoding rat CD9, we used the PCR with nondegenerate oligonucleotide primers corresponding to the NH₂-terminal and COOH-terminal sequences of human/bovine CD9. Amplifications were performed using cDNA templates derived from adult rat sciatic nerve or superior cervical sympathetic ganglia mRNA. In both cases, 690 bp products were obtained (data not shown). This corresponds to the expected size for a cDNA clone encoding the entire rat CD9 protein based upon the nucleotide sequences of human and bovine CD9. Two cDNAs obtained using the sciatic nerve (ScN) template, full ScN#13 and #42, as well as two cDNAs obtained using the superior cervical sympathetic ganglia (SCG) template, full SCG#3 and #8, were sequenced in both directions (see Materials and Methods), and found to be nearly identical (see Materials and Methods). In addition, we have isolated and characterized three full length cDNAs encoding rat CD9 from a postnatal day 9 rat

sciatic nerve cDNA library. The sequences of these clones precisely match those of the PCR products (data not shown).

The rat protein is 95% identical to mouse CD9, and 93%, 92%, and 83% identical to the human, monkey, and bovine proteins, respectively (Fig. 2). Rat, mouse, and bovine CD9 each consist of 226 amino acid residues, while human and monkey CD9 are comprised of 228 amino acids. Only rat and mouse CD9 contain a string of seven amino acids between positions 120 and 126, ELQEFYK, which is identical to the sequence of a tryptic peptide generated from the affinity-purified 26 kDa protein (Fig. 2 and Kaprielian and Patterson, 1993). At position 50 in the rat sequence there exists one putative, N-linked glycosylation site (NHS). A similar site is present in the mouse, human, monkey and bovine sequences; each of these sequences, however, possesses additional asparagine residues immediately before the consensus asparagine. Mouse and bovine CD9 contain one, while human and monkey CD9 contain two additional asparagines (Fig. 2). Consistent with previous observations (Rubinstein et al., 1993b), the majority of interspecies amino acid differences in the CD9 protein are present in the large extracellular domain (Fig. 2).

The 26 kDa ROCA antigen is rat CD9

To confirm the identity of the 26 kDa ROCA antigen, the rat CD9 cDNA clone, full ScN #13, was subcloned into the pcDNA1/neo expression vector (Invitrogen) and introduced into CHO cells. Multiple, stably transfected, clonal CHO cell lines were isolated through G418 selection. ROCA1, ROCA2, and B2C11 each immunoprecipitate a 26 kDa protein from detergent extracts of such cell lines (Fig. 3A). Isotype-matched mAbs do not immunoprecipitate CD9 (data not shown). In addition, ROCA2 and B2C11, but not ROCA1, strongly and uniformly

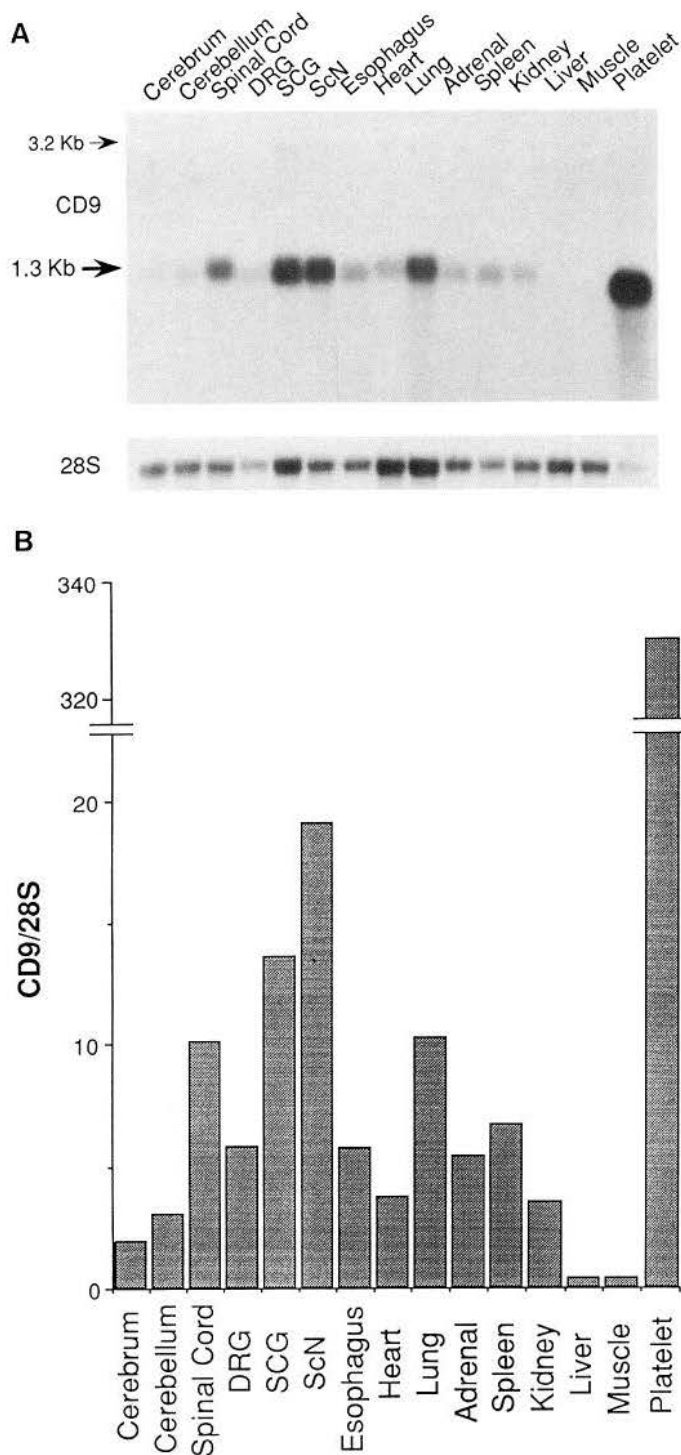


Figure 5. Rat CD9 mRNA is expressed by a wide range of neural and non-neural tissues. *A*, Northern blot analysis was performed using 2 μ g of total RNA derived from various adult rat tissues and 32 P-labeled Full ScN #13 (rat CD9) and 28S cDNA probes. The top panel shows the hybridization of the rat CD9 probe to a 1.3 kb species present in many tissues, and the bottom panel shows the hybridization of the 28S probe to all tissues. The apparent size difference between platelet CD9 mRNA and CD9 mRNAs in other tissues is due to frowning of this particular gel and the placement of the platelet lane at the extreme edge of the gel. *B*, The radioactivity in all lanes was quantified using a Phosphorimager, and plotted as the ratio of CD9 mRNA to 28S rRNA versus tissue type.

label the surfaces of these same cells (Fig. 3*B*). None of our anti-rat CD9 mAbs bind the surfaces of (Fig. 3*C*), or immunoprecipitate any protein from (data not shown), the mock-transfected CHO cells (Fig. 3*C*).

To prove that ROCA2 and B2C11 recognize the large, putative extracellular domain of CD9, we generated a fusion protein (see Materials and Methods) containing residues 111–190 of rat CD9. Consistent with the fact that ROCA2 and B2C11 bind living cells expressing the entire rat CD9 protein, ROCA2 and B2C11, but not ROCA1, bind this fusion protein (Fig. 4).

Expression of rat CD9 mRNA

Tissue distribution. Northern blot analysis was performed using total RNA from various adult rat tissues and the full ScN #13 rat CD9 cDNA probe. This probe identifies a 1.3 kb RNA band in a wide range of tissues (Fig. 5*A*). For each tissue, the ratio of CD9 mRNA to 28S rRNA was calculated (Fig. 5*B*). Based upon this analysis, adult rat platelets are the most abundant source of CD9 mRNA. This is consistent with the known distribution of CD9 in other species. Superior cervical ganglia (SCG), sciatic nerve (ScN) and spinal cord all express high levels of CD9 mRNA. Low, but clearly detectable levels are present in dorsal root ganglia (DRG) and cerebellum, but very little signal is detected in the cerebrum. While significant levels of CD9 mRNA are present in a number of non-neural tissues such as esophagus, heart, lung, adrenal, spleen, and kidney, no hybridization was observed to liver or skeletal muscle RNA. The rat CD9 probe also hybridizes to a minor, 3.2 kb species in superior cervical ganglia and sciatic nerve RNA (Fig. 5*A*; see Discussion).

Cell type specific expression. *In situ* hybridization studies were performed to unambiguously determine whether neurons as well as glia express CD9 mRNA. Digoxigenin-labeled antisense and sense RNA probes derived from the full ScN #13 cDNA were hybridized to sections of selected neural and non-neural tissues. The cytoplasm of adult rat SCG and DRG neurons is clearly labeled by the antisense probe (Fig. 6*a,c*). While most of the neurons present within SCG sections are labeled by the rat CD9 probe, only a small subset of DRG neurons detectably express CD9 mRNA. This finding is consistent with a significantly lower level of CD9 mRNA in the DRG as compared to the SCG, as determined by Northern analysis. Schwann cells present within sections of adult rat peripheral nerve also clearly express CD9 mRNA (Fig. 6*b*). The identification of these cells as Schwann cells is based upon (1) labeling of very similar appearing cells using Po and MBP RNA probes (data not shown), (2) the bipolar, longitudinal morphology of the labeled cells, and (3) the large number of CD9⁺ cells. In addition, the cytoplasm of chromaffin cells in the adrenal medulla (Fig. 6*d*) and epithelial cells in the esophagus (data not shown) strongly label with the antisense CD9 probe. In all cases, no hybridization was detected with the sense probe (data not shown).

Positional and developmental expression. We also examined the rostrocaudal distribution of CD9 mRNA. Northern analysis detects essentially equivalent levels of the 1.3 kb CD9 mRNA (normalized to the amount of myelin basic protein (MBP) mRNA) in adult rat intercostal nerves taken from various rostrocaudal levels (Fig. 7). Similarly, the number of Schwann cells expressing rat CD9 mRNA does not appear to vary significantly between rostral and caudal nerves (data not shown).

Northern and *in situ* analyses were also used to determine if CD9 mRNA expression is developmentally regulated in postnatal rat ScNs. We performed similar analyses with probes spe-

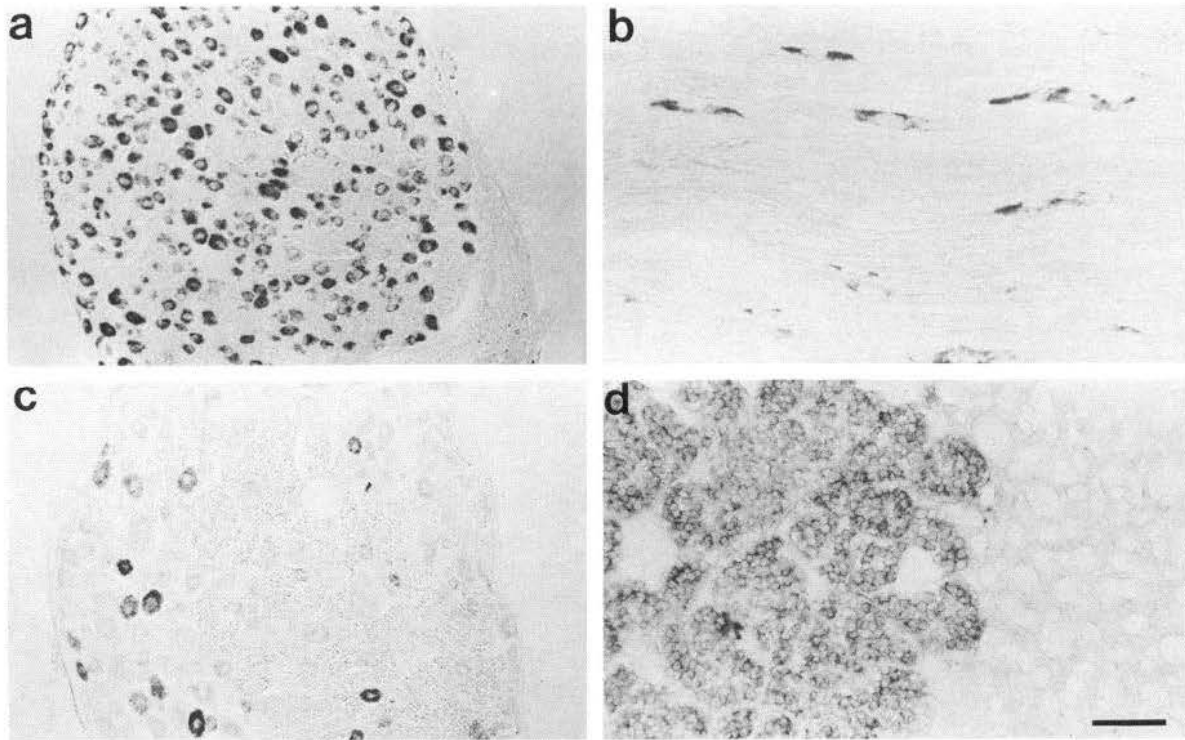


Figure 6. Rat CD9 mRNA is expressed by peripheral neurons, Schwann cells and chromaffin cells. Digoxigenin-labeled antisense RNA probes derived from the Full ScN #13 cDNA were hybridized to cryosections of adult rat SCG (*a*), sciatic nerve (*b*), DRG (*c*), and adrenal gland (*d*). The cytoplasm of neurons present within the SCG and DRG sections (*a*, *c*) and bipolar Schwann cells in the sciatic nerve section (*b*) are all brightly labeled. The left portion of the adrenal gland section (*d*) contains strongly labeled chromaffin cells of the adrenal medulla, while the right portion shows the unlabeled cortex.

cific for Po and MBP, two myelin genes whose expression is known to be developmentally regulated in these nerves (Wiggins et al., 1975; Lees and Brostoff, 1984; Lemke and Axel, 1985; Stahl et al., 1990). Northern analysis shows that the mRNA expression of all three genes dramatically rises from a low, but clearly detectable level in newborn sciatic nerves, to maximum levels by postnatal day 14 (Fig. 8*A*). By day 60, CD9, MBP, and Po mRNA expression is downregulated to adult levels. *In situ* hybridization performed with the full ScN #13 CD9 antisense probe confirms these results. While only a small number of scattered Schwann cells express CD9 mRNA in the newborn ScN, large numbers are strongly labeled by postnatal days 7 and 14 (Fig. 8*B*). By day 60, the number of labeled Schwann cells is reduced to adult levels. Similar results were obtained with a MBP antisense probe (data not shown).

Discussion

We previously generated the mAb, ROCA1, which recognizes an epitope on a 26 kDa cell surface protein that is preferentially accessible in rostral intercostal nerves and rostral sympathetic ganglia (Suzue et al., 1990; Kaprielian and Patterson, 1993). Two other mAbs, ROCA2 and B2C11, also recognize the 26 kDa protein, but do not detect a rostrocaudal, immunohistochemical gradient. Amino acid sequences obtained from the affinity-purified protein suggested that it could be the rat homolog of CD9 (Kaprielian and Patterson, 1993). We present here the isolation of cDNA clones encoding rat CD9 from peripheral nerves and ganglia, and demonstrate that the ROCA cell surface protein is, in fact, rat CD9. We also show that rat CD9 mRNA is expressed at high levels in peripheral neurons

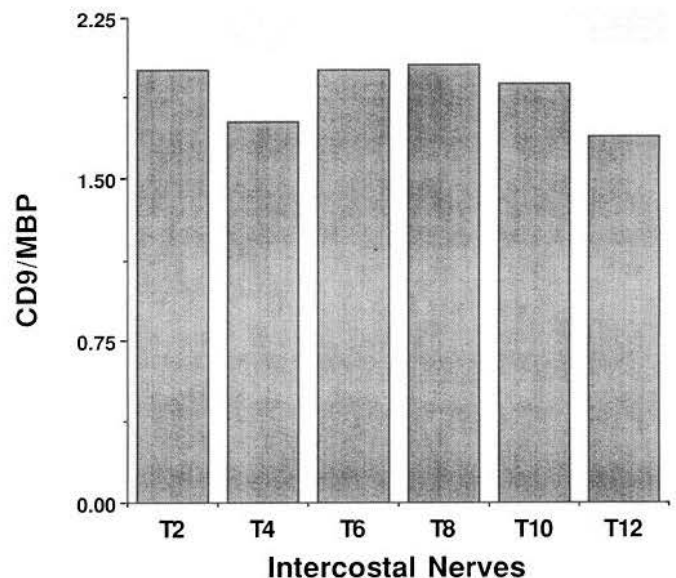


Figure 7. The expression of rat CD9 mRNA is not graded along the rostrocaudal axis. Northern blot analysis was performed using 2 μ g of total RNA derived from adult rat intercostal nerves taken from various rostrocaudal levels, and 32 P-labeled Full ScN #13 (rat CD9) and MBP cDNA probes. The identical transfer was probed with each cDNA in succession after removal of previously bound radioactivity. The radioactivity representing the hybridization of each probe was quantified using a Phosphorimager and the ratio of CD9 mRNA to MBP mRNA plotted versus rostrocaudal position.

and Schwann cells, as well as in certain parts of the central nervous system. In addition, we demonstrate that while CD9 mRNA is not positionally regulated in intercostal nerves, it is coordinately regulated with myelin genes during postnatal ScN development.

The ROCA cell surface 26 kDa protein is rat CD9

Using the PCR, as well as direct cDNA library screening, we obtained cDNA clones representing the probable entire coding region of rat CD9 from SCG and ScN. The high degree of amino acid identity between the deduced protein sequence of the rat form and the mouse (95%), human (93%), monkey (92%), and bovine (83%) forms strongly supports the identification of these cDNAs as rat CD9. Our CD9 mAbs bind the cell surface and fail to cross-react across species. The larger, putative extracellular loop that contains the majority of interspecies amino acid differences (Martin-Alonso et al., 1992; Reynolds et al., 1992; Wright et al., 1993), also contains the epitopes recognized by ROCA2 and B2C11, as shown by positive binding to immunoblots of a fusion protein containing residues 111–190 of the rat protein. Interestingly, ROCA1 does not bind this fusion protein, even though it binds the full-length protein very well (Fig. 3A). This result, coupled with the finding that ROCA1 can bind to the complete fusion protein on immunoblots and to intact primary cells, suggests that the position-dependent ROCA1 epitope resides on the smaller extracellular loop (residues 36–55) of CD9.

The deduced rat CD9 protein sequence contains the same string of seven amino acids, ELQEFYK, as one of the tryptic peptides generated from the affinity purified 26 kDa protein (Kaprielian and Patterson, 1993). The N-terminal 14 amino acids (deduced from the cDNA) of the rat protein, PVKGGSKCIKYLLF, are similar to those obtained by sequencing the N-terminus of the affinity-purified ScN protein, XVKGGIDEVFYLLF (Kaprielian and Patterson, 1993). All 12 of the rat CD9 cDNAs, contain the amino acid string, SKCIK, not IDEVF, between positions 6 and 10 within this string. This discrepancy may be due to protein sequencing errors or to the existence of two forms of rat CD9 with different N-terminal sequences. Support for the protein sequencing error explanation comes from previous attempts to sequence the N-terminus of human CD9; three different groups failed to unambiguously determine the identity of all five residues (Higashara et al., 1990; Boucheix et al., 1991; Lanza et al., 1991).

We performed additional PCR reactions to search for a form of CD9 containing the amino acid string IDEVF from amino acid position 7 to 11. Sequence analysis of 23 distinct clones identified either the same form of rat CD9 that we report here (N-terminus: MPVKGGSKCIK) or nonsense sequences (data not shown). Despite these results, however, we cannot rule out the existence of another, perhaps alternatively spliced, form of rat CD9.

Immunoprecipitation of a 26 kDa protein by ROCA1, ROCA2, and B2C11 from detergent extracts of CHO cells stably transfected with a rat CD9 cDNA confirms the identity of the ROCA cell surface antigen as CD9. Interestingly, ROCA1, unlike ROCA2 and B2C11, does not label the surfaces of the transfected cells. ROCA1 does not bind the surface of a variety of other cell types that are strongly labeled with ROCA2 and B2C11 (Hadjiargyrou and Patterson, 1994, and unpublished observations), but does bind to the surfaces of primary Schwann cells (Tole, unpublished observations). Together with our previous

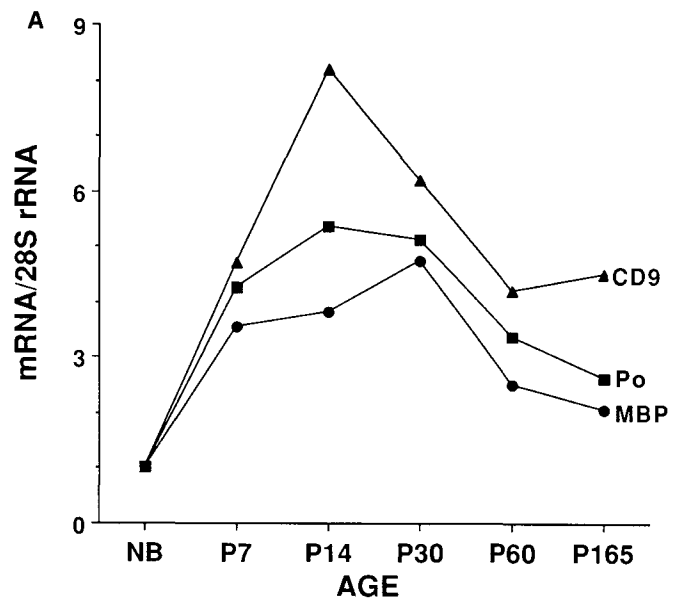


Figure 8. The expression of rat CD9 mRNA is developmentally regulated in postnatal sciatic nerves. *A*, Northern blot analysis was performed using 2 μ g of total RNA derived from sciatic nerves taken from postnatal rats of various ages, and 32 P-labeled Full ScN #13 (rat CD9), MBP, Po, and 28S cDNA probes. The identical transfer was probed with each cDNA in succession after removal of previously-bound radioactivity. The radioactivity representing the hybridization of each probe was quantified using a Phosphorimager and the ratio of the various mRNAs to 28S rRNA plotted versus developmental age. *B*, Digoxigenin-labeled antisense RNA probes derived from the Full ScN #13 cDNA were hybridized to cryosections of developing postnatal rat sciatic nerves. The number of Schwann cells expressing rat CD9 significantly increases by postnatal day 7, and then decreases to adult levels by postnatal day 60.

in vivo observations (Kaprielian and Patterson, 1993), these results suggest that the ROCA1 epitope may not always exist in an accessible state on the cell surface. In this regard, the transfected CHO cells may represent a model system for further study of epitope masking on CD9.

Expression of rat CD9 mRNA

Tissue and cell type specific expression. We detect a major 1.3 kb band of rat CD9 mRNA in many, but not all adult tissues. Of the tissues examined, only liver and skeletal muscle contain no detectable rat CD9 mRNA. The significant levels of CD9 mRNA present in certain neural tissues, including adult rat peripheral nerves and ganglia, spinal cord, and cerebellum, are consistent with the isolation of rat CD9 cDNAs from adult SCG and ScN DNA templates. Other studies have identified a similar size mRNA band in Northern blots using CD9 cDNA probes (Boucheix et al., 1991; Martin-Alonso et al., 1992). However, an extensive analysis of CD9 mRNA tissue distribution has not previously been described. The rat CD9 probe also hybridized weakly to a 3.2 kb mRNA band. While the nature of this species is not known, this band is present in a wide variety of tissues and cell lines (data not shown).

In situ hybridization shows that peripheral neurons, glia and chromaffin cells express rat CD9 mRNA. In particular, neurons in SCG and DRG, Schwann cells in ScNs, and chromaffin cells in the adrenal medulla are all strongly labeled by the CD9 antisense probe. Neuronal labeling is clearly detectable in early postnatal sympathetic and sensory ganglia (data not shown) and

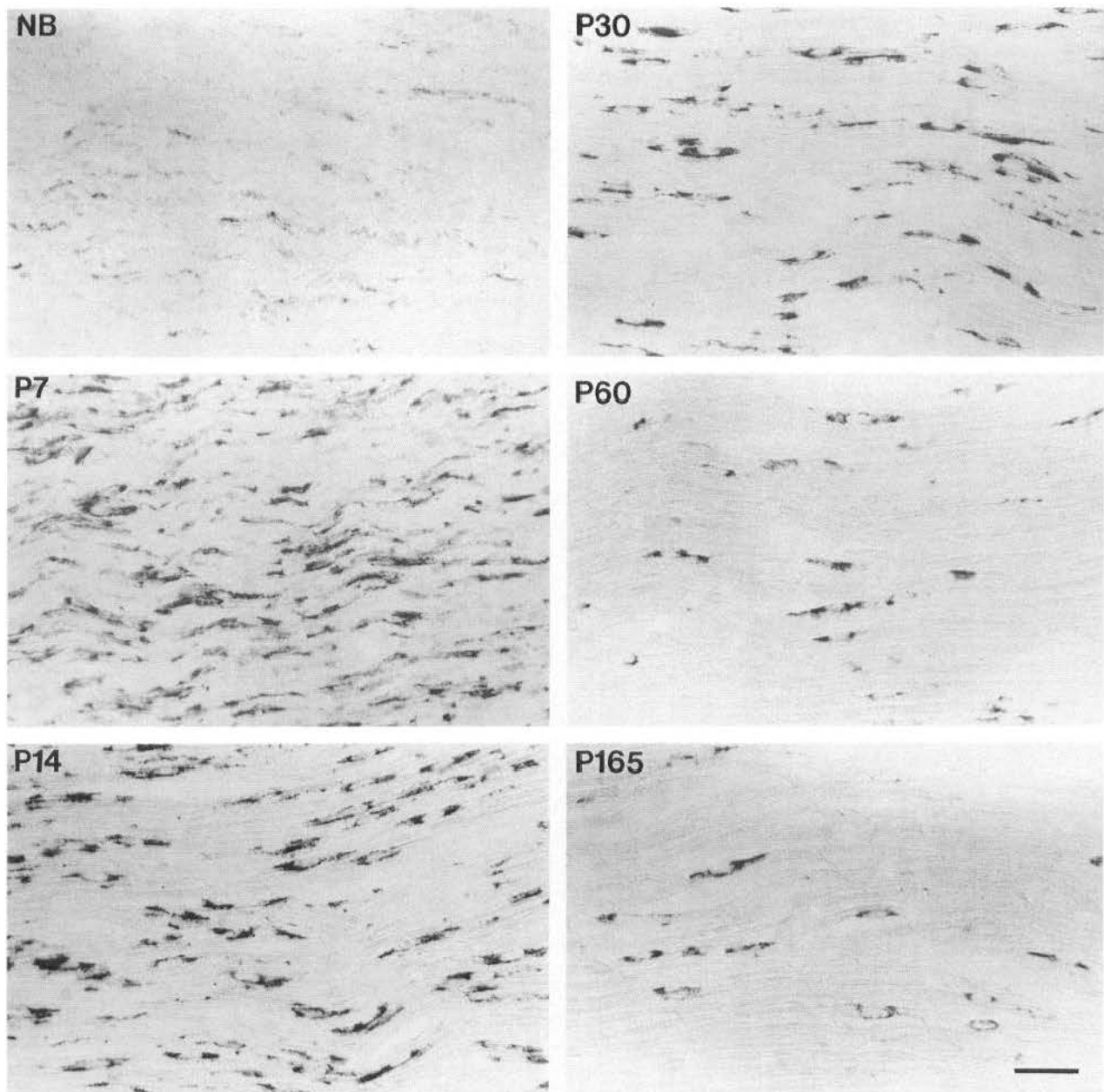
B

Figure 8. continued.

is maintained into adulthood. We have previously shown that satellite cells in sections of adult SCG are DRG are labeled by ROCA1 and 2 (Suzue et al., 1990; Kaprielian and Patterson, 1993; Tole and Patterson, 1993), as well as B2C11 (unpublished observations). We cannot, however, unambiguously detect label in the cytoplasm of ganglionic satellite cells in our *in situ* analysis. This may reflect the presence of lower levels of CD9 mRNA in satellite cells than in neurons. Taken together, the northern blot and *in situ* hybridization results confirm and extend previous immunoblot and immunohistochemical analyses demonstrating that rat peripheral neurons, Schwann cells, and chromaffin cells all express CD9 (Kaprielian and Patterson, 1993; Tole and Patterson, 1993). To our knowledge, this is the first

study utilizing *in situ* hybridization to localize CD9 mRNA in any tissue.

Positional and developmental expression. Northern blot analysis shows that equivalent levels of CD9 mRNA are present in rostral and caudal intercostal nerves. This result is consistent with previous immunoblot analyses demonstrating that both ROCA1 and ROCA2, as well as B2C11 (Z. Kaprielian, unpublished observations), detect uniform levels of the 26 kDa, rat CD9 protein in rostral and caudal intercostal nerves (Kaprielian and Patterson, 1993). These findings all support the notion that ROCA1 binding reflects the presence of a positionally graded epitope, rather than a rostrocaudal gradient of CD9 protein. Of course, the formal possibility remains, that ROCA1 recognizes

an as yet unidentified third antigen (in addition to CD9 and peripherin) whose distribution may directly account for the immunohistochemical gradient.

Northern blot and *in situ* hybridization analyses shows that the expression of CD9 mRNA is significantly upregulated during early postnatal SCN development, and downregulated in the adult. This time course suggests that CD9 and peripheral myelin gene expression (Wiggins et al., 1975; Lees and Brostoff, 1984; Lemke and Axel, 1985; Stahl et al., 1990) are coordinately regulated during postnatal development. Together with the expression of the CD9 protein on neuronal as well as Schwann cell surfaces, these developmental data further suggest a role for CD9 in mediating Schwann cell-axon interactions during myelination. Moreover, the results of antibody perturbation experiments strongly suggest a role for CD9 in neural cell adhesion (Hadjigryrou and Patterson, 1994).

Interestingly, the developmental expression of CD9 also parallels that of another peripheral myelin gene PMP-22 (Welcher et al., 1991; Snipes et al., 1992). PMP-22 is the target of mutations that result in the trembler phenotype in mice and in Charcot-Marie-Tooth disease (CMT) Type 1a in humans (Snipes et al., 1993). Although PMP-22 bears no sequence homology with members of the CD9 tetraspan family, this protein has also been implicated in growth regulation and possesses four putative membrane-spanning domains (Lemke, 1993; Snipes et al., 1993; Suter et al., 1993). In this regard, it is interesting to note that another four transmembrane spanning domain molecule, connexin 32 (Cx32), has recently been shown, like MBP, P0, and PMP-22, to be downregulated in transected sciatic nerves (S. Scherer, personal communication). Moreover, patients with X-linked CMT possess mutations in the gene encoding Cx32 (Bergoffen et al., 1993). Thus, it seems possible that proteins containing the four transmembrane domain structural motif may play important roles in peripheral nerve myelination.

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